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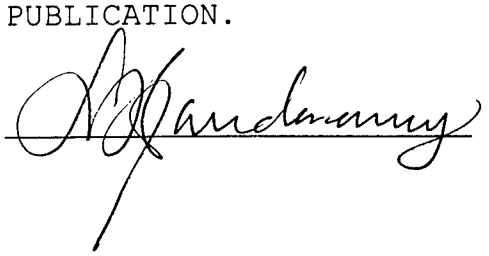

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13. ABSTRACT *(Maximum 200 Words)*

New strategies to improve the outcome of patients with metastatic breast cancer must address the issue of residual chemotherapy-resistant cells. Immunotherapy offers the potential of manipulating effector cells to generate an enhanced anti-tumor effect. From initial data demonstrating that natural killer (NK) cells require interleukin-2 (IL-2) in order to lyse breast cancer targets, we have now explored the mechanisms which mediate tumor killing. Our research has demonstrated that NK cell killing is variable and partially dependent on recognition through ICAM-1 and CD18; however, upregulation of ICAM-1 expression by cytokines does not enhance NK killing. CD58 antibody-dependent cytotoxicity (ADCC) is dependent on FcRγIII (CD16), and also on CD2 and ICAM-1/CD18. Herceptin mediates ADCC through a different mechanism. Our data show that multiple mechanisms are involved in NK cell lysis of breast cancer targets, that none of the targets are inherently resistant to killing, and that two distinct mechanisms of ADCC can target immunotherapy to breast cancer cells.

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Linda V Bonny MD 9/30/99

PI - Signature Date

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INTRODUCTION

Breast cancer is the most common malignant neoplasm in women and is the second leading cause of cancer-related death in the United States. *In vitro*, preclinical, and clinical trials have shown that chemotherapy dose intensity is an important component of therapy. High dose chemotherapy with autologous peripheral stem cell support permits high-dose intensive chemotherapy treatment and may be beneficial for certain patients with advanced breast cancer. However, a high rate of relapse accounts for the majority of patients' deaths following transplantation. Patients typically relapse at sites of prior disease, suggesting that minimal residual disease may not have been eradicated by the preparative regimen.

Novel anti-tumor strategies are clearly needed. We hypothesize that immunotherapy used in a minimal residual disease setting after transplantation may serve as a noncross-resistant therapy to prevent relapse. Although NK cells are among the first immune effectors to reconstitute after stem cell transplantation, resting NK cells do not exhibit activity against breast cancer targets until they are activated with exogenous IL-2. We demonstrated that interleukin-2 (IL-2) activated natural killer (NK) cells were able to lyse breast cancer cell targets, in part through intercellular adhesion molecule-1 (ICAM-1) expression, but that other molecules must also be involved in tumor cell recognition.

Following the first year of this research project, we demonstrated that multiple mechanisms are involved in NK killing of breast cancer targets, including β2 integrins, CD2, and LFA-3 (CD58) mediated antibody-dependent cellular cytotoxicity (ADCC). This year we have confirmed these initial observations, and have further extended our experiments to include elucidation of the LFA-3 mediated ADCC by exploring another antibody, Trastuzumab (Herceptin). Trastuzumab mediated ADCC against all the HER2/neu positive breast cancer targets. Unlike CD58 antibody-mediated ADCC, Trastuzumab ADCC was minimally affected by blocking antibodies to CD2 or ICAM-1/CD18, which suggests a different mechanism of action.

BODY

Preliminary results reported in first annual report which have been confirmed, and represent key accomplishments in the progress of this project:

1. NK cells require IL-2 activation to kill breast cancer targets. Determination of the mechanisms involved in NK killing of breast cancer targets formed the basis for this project.

2. We originally hypothesized that sensitivity to lysis by IL-2 activated NK cells would directly correlate with relative expression of ICAM-1 on targets. However, our results do not support this premise. There was no correlation between surface expression of ICAM-1 and target sensitivity to NK cell lysis, and induction of ICAM-1 on targets by cytokines failed to make them more susceptible to lysis. This data has been confirmed. As our data did not support our original premise that upregulation of ICAM-1 surface expression by cytokines plays a significant role in activated NK cell killing, we elected to explore mechanisms of NK killing involving other potential recognition molecules as part of Technical Objective 2 (Months 12-48), and not to pursue identification of cis sequences or protein factors regulating ICAM-1 expression as outlined in the original statement of work, Tasks 2 and 3.

3. The interaction of CD2 on NK cells with its ligand, CD58 (LFA-3) on breast cancer cells was investigated. CD2 antibody did not significantly effect a change in specific lysis. In contrast, addition of CD58 antibody (AICD58) to targets consistently increased killing of breast cancer targets MB-231, BT-20 and SKBR-3. We have performed additional control experiments since last year which demonstrated that the addition of the CD58 antibody alone to targets without effectors did not result in lysis, lending further support to our hypothesis that CD58 antibody may function through antibody dependent cellular cytotoxicity (ADCC).

4. Consistent with ADCC, the CD58 (AICD58) antibody effects were independent of IL-2 activation and NK cell CD16 (FcRγIII) was required in the process. We used unique differences between mature NK cells and those derived from long-term cultures of marrow progenitors to generate NK cells that were CD16 negative. We have shown that these cells exhibit characteristic lysis of K562 targets demonstrating that their lytic machinery is intact. The failure of the CD58 (AICD58) antibody to enhance killing by the marrow progenitor-derived NK cells demonstrates a requirement for CD16.

5. Anti-CD-58-mediated ADCC is clone specific, as another CD58 clone (BRIC-5) resulted in no difference in lysis of breast cancer targets by IL-2 activated NK cells. As both CD58 antibodies were isotype IgG2a, the inability of clone BRIC-5 to mediate ADCC may be due to epitope specificity or to some characteristic of tertiary structure.

New accomplishments in the progress of this project during the past 12 months:

We have continued to define how ADCC by breast cancer is mediated and the potential role of accessory cell molecules, in a series of experiments as described below.

1. Trastuzumab (Herceptin) mediates ADCC

To further explore the finding of CD58 antibody mediating ADCC, we tested another antibody that mediates ADCC. Herceptin is a humanized antibody against

HER/neu2 which has been engineered by inserting the complementary determining regions of a murine antibody (clone 4D5) into the framework of a consensus human IgG1. The HER2/neu murine antibody (clone 2G11, IgG1) did not mediate ADCC. In contrast, Herceptin added to normal CD56+/CD3- NK cells significantly enhanced killing of all breast cancer targets except for MDA-MB-231, the target with the lowest HER2/neu expression (Table 1, Figure 1). Titration experiments with the Herceptin antibody and the SKBR-3 target, the target with the highest expression of HER2/neu, showed enhanced lysis down to an antibody concentration of 0.01 ug/mL (n=2), which was the concentration used in subsequent ADCC blocking experiments.

Table 1. Mean channel fluorescence of HER2/neu on breast cancer cell lines

Cell line	HER2/neu
MCF-7	62
T47D	83
MDA-MB-231	54
BT-20	105
SKBR-3	1676

Mean channel fluorescence (MCF) of the isotype control was between 6 and 22 for all samples

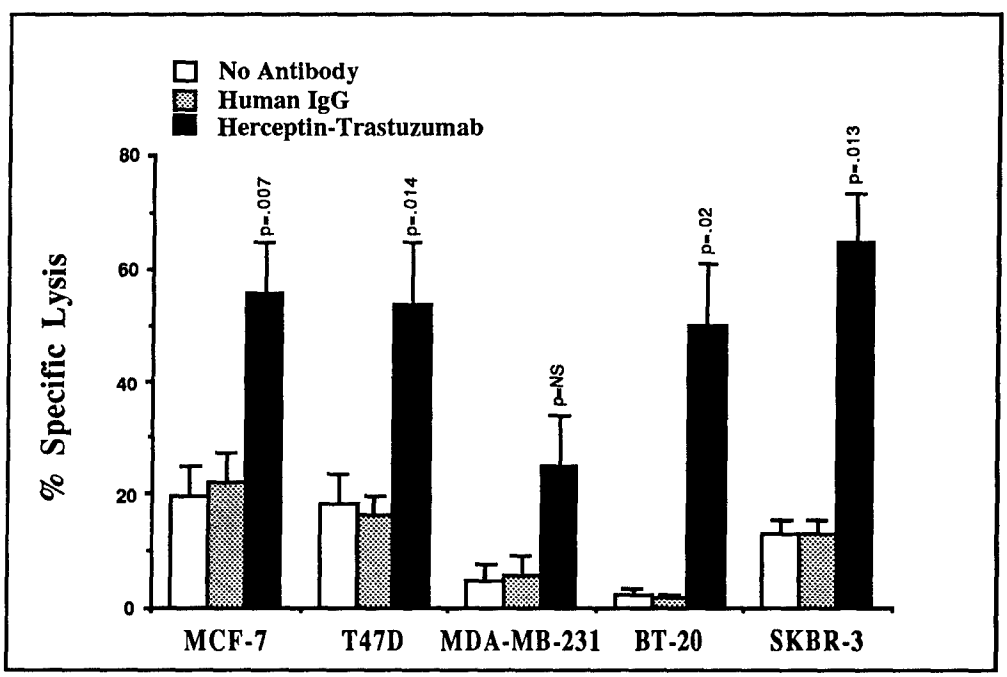

Figure 1. Incubation of breast cancer targets with Herceptin (humanized anti-HER2/neu) mediates antibody-dependent cellular cytotoxicity. Cytotoxicity was performed without antibody or with the addition of human IgG or Herceptin at 10 ug/mL (n=4 in triplicate). Cytotoxicity with Herceptin was compared to the human IgG control.

2. Herceptin mediated killing is FcRγIII (CD16) dependent

Marrow-derived CD16- NK cells did not augment killing of SKBR-3 targets in the presence of Herceptin. Similar to CD58 (AICD58) ADCC, Herceptin augmented killing by resting blood NK cells was also FcRγIII (CD16) dependent as shown using blocking antibodies (Figure 2).

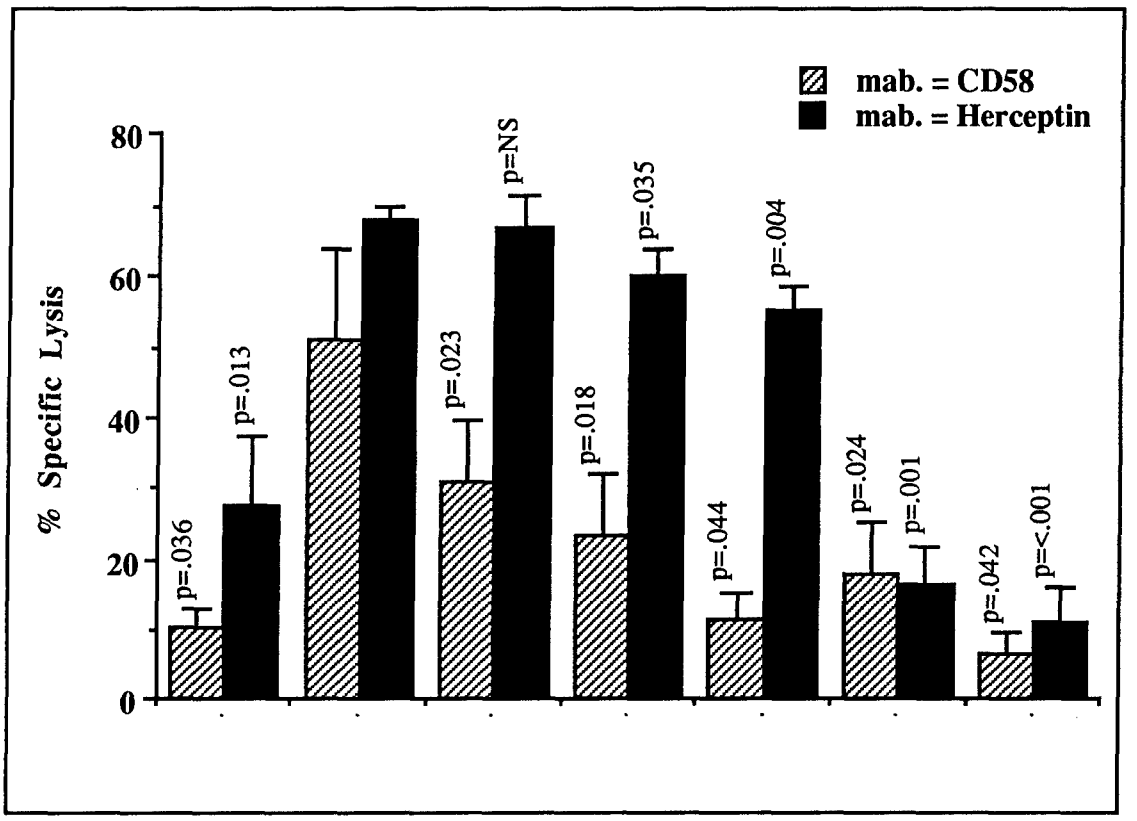

Figure 2. CD58 antibody-mediated antibody-dependent cellular cytotoxicity (ADCC) but not Herceptin-mediated ADCC is decreased by blocking ICAM-1/CD18 and CD2. Cytotoxicity was performed in the presence or absence of blocking antibodies as indicated (n=4 donors in triplicate). All p values listed are compared to the addition of monoclonal antibody (mab) alone (*).

3. Herceptin mediated killing is only slightly blocked by antibodies to CD2 and ICAM-1/CD18

In contrast to CD58 (AICD58) ADCC, which was decreased by nearly 50% by CD2 or ICAM-1/CD18, these same blocking antibodies had less of an effect on Herceptin ADCC (Figure 2). Whereas blocking both CD2 and ICAM-1/CD18 completely abrogated CD58 (AICD58) ADCC, ADCC with Herceptin was only slightly blocked with the same combination of antibodies.

4. CD58-mediated ADCC but not Herceptin-mediated ADCC is dependent on CD2

Although both antibodies [CD58(AICD58) and Herceptin] result in CD16-dependent killing, blocking experiments suggest different interactions with accessory receptor/ligand pairs. CD58 (AICD58)-mediated ADCC appears to be CD2 dependent, whereas Herceptin ADCC is minimally affected by blocking CD2. To further test this, we

used a subset of NK cells that is CD56 and CD16 positive but CD2 negative. This subset, which generally comprises 10 to 40% of normal blood NK cells, was purified by flow cytometry (Figure 3A). Secondary staining of CD56+/CD2- sorted NK cells showed that greater than 80% expressed CD16. CD56+/CD16+/CD2- NK cells were still able to augment target lysis of Herceptin-treated SKBR-3 targets, which suggests a CD2-independent mechanism of ADCC signaling through CD16. In contrast CD56+/CD16+/CD2- NK cells did not lyse CD58 (AICD58) antibody-treated BT-20 targets, which confirms the CD2 dependence of this ADCC and the lack of triggering through CD16 alone (Figure 3B).

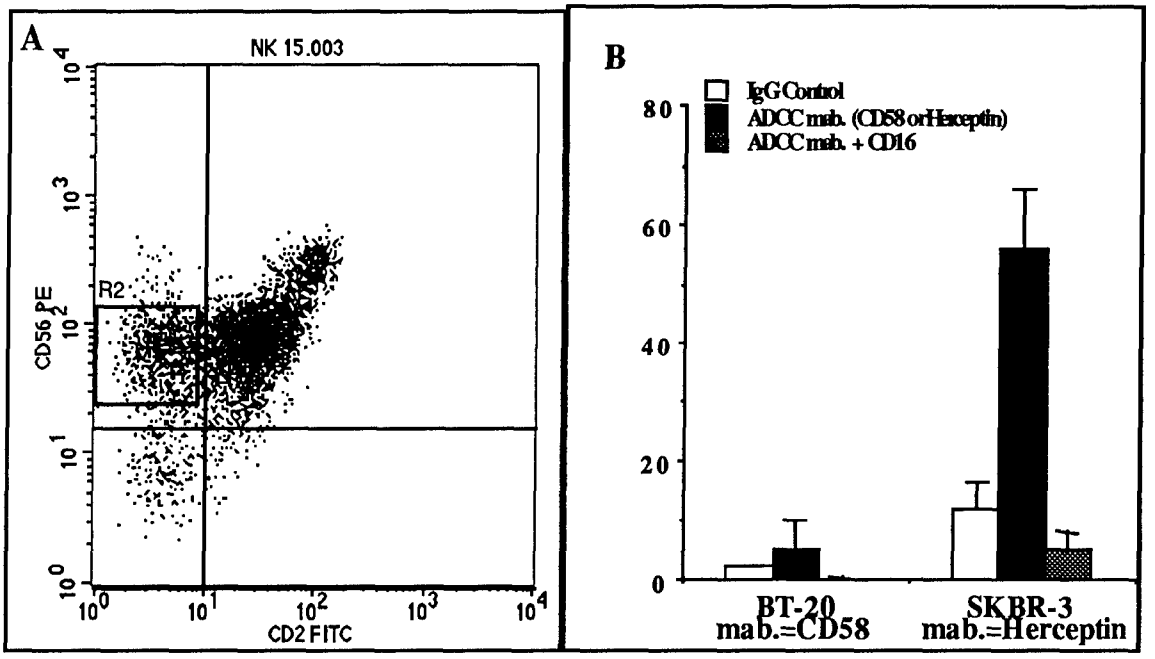

Figure 3. CD58-mediated antibody-dependent cellular cytotoxicity (ADCC) but not Herceptin-mediated ADCC is dependent on CD2. (A) CD56+/CD2- NK cells were sorted by flow cytometry. An example of a representative sort with the collection window designated R2 is shown. (B) CD56+/CD2- NK cells, which are predominantly CD16 positive, were tested in cytotoxicity assays using the breast cancer target and antibodies as indicated. The CD2-NK cells exhibited CD16-dependent killing of Herceptin-treated SKBR-3 targets but had no effect on CD58 (AICD58) antibody-treated BT-20 targets.

5. Technical Objective 3: HLA class I locus specific expression, regulation, and role in cytotoxicity

Multiple structures and variants of HLA Class I recognizing receptors are found on all NK cells, although their physiologic relevance is uncertain. Early observations noted that the sensitivity of tumor targets to lysis by NK was inversely related to the levels of HLA class I molecules on the target cell surface.

We initially focused on interferon γ, as treatment of breast cancer cell lines with this cytokine resulted in the greatest fold increase in class I expression (TNF, IL-1 had minimal effects), and as there was theoretical therapeutic potential in the treatment of women undergoing autologous transplantation for breast cancer with interferon, as others have done in an effort to augment cyclosporine induced graft-versus-host-disease.

The metastatic breast cancer cell line, SKBR-3, exhibited the lowest basal expression of surface HLA Class I, and exhibited a 3-fold increase in surface expression with interferon γ treatment (from a mean channel fluorescence of 222 to 607). Interferon-γ induced HLA Class I expression on SKBR-3 targets resulted in resistance to lysis by NK cells. As will be described below, we plan to begin investigating immunotherapeutic approaches utilizing antigen presenting cells and cytotoxic T-lymphocytes to enhance specificity of tumor cell killing. As HLA Class I expression may be more critical to T-lymphocyte recognition of tumor cells, we will pursue this objective in the next 2 years depending on results of future studies.

New Objectives for years 03 and 04:

1. NK cell function in response to IL-2 + Stem Cell Factor

With our data demonstrating Herceptin medicated ADCC, we have translated these findings into a Phase I clinical trial of the combination of IL-2 and Herceptin in patients with metastatic breast cancer. Our objectives in this trial are to determine if the combination of IL-2 and Herceptin can be safely given, and to perform correlative laboratory studies to determine NK cytotoxicity against breast cancer cells. Laboratory studies will test NK cell function before and after initiation of IL-2 + Herceptin therapy.

2. NK, T-cell, and dendritic cell funtion in response to IL-2 + Stem Cell Factor

We have based our research efforts to date on our initial data which demonstrated enhanced lysis of breast cancer targets by IL-2 activated NK cells. While this approach to immunotherapy holds promise and deserves further exploration, we also recognize that it lacks specificity. Immunotherapy may be more efficacious by incorporating approaches which also target cytotoxic T-cells (CTL). Antigen presenting cells (APC), of which dendritic cells are the most potent, are critical in educating T-cells to recognize cancer.

Based on the known distribution of c-kit receptors on a subset of $CD56^+$ bright NK cells and other primitive lymphoid progenitors, we have initiated a Phase I clinical trial incorporating IL-2 along with SCF (Stem Cell Factor, c-kit ligand) as post-transplant immunotherapy in patients with chemosensitive metastatic breast cancer. Correlative laboratory studies will test NK cell, T-cell and dendritic cell function before and after initiation of cytokine therapy. *In vivo* mobilized dendritic cells will be compared with dendritic cells derived from monocytes (after culture with GM-CSF, IL-2, ± TNF) and CD34+ cells (after culture with SCF, Flt-3 Ligand, GM-CSF, IL-4, ± TNF).

We anticipate that these studies will result in important information on how best to elicit immune responses from vaccines, as we hypothesize that a strategy using both lymphoid and antigen presenting cell (APC) stimulation will be optimal for this purpose. We may demonstrate that dendritic cell mobilization *in vivo* results in a mature phenotype which may be good for antigen uptake but weak in terms of costimulatory function. We will determine whether an *ex vivo* culture step is necessary after *in vivo* mobilization and how this compares to a more cumbersome generation of dendritic cells from monocytes or $CD34^+$ cells.

3. Vaccination with tetanus toxoid and Keyhole Limpet Hemocyanin (KLH) to assess antigen specific responses

To improve the efficacy of immune therapies, we are interested in tumor vaccines to more specifically target therapy. For a vaccine to be effective, antigen must be taken up and processed by antigen presenting cells, and then presented to specific T-cells which cooperate with B-cells which in turn mediate the antibody response. There are many

questions about how to best manipulate the immune system to enhance vaccines. In a clinical protocol, we will be testing the immune response using two known safe vaccines: tatanus toxoid which is routinely given to the general population, and Keyhoe Limpet Hemocyanin (KLH), an antigen which is immunogenic in humans. The advantage of KLH is that it measures responses to an antigen that people would unlikely have been exposed to in the past (called a neo-antigen). After recovery from autologous transplantation for breast cancer, patients will receive vaccination only once with tetanus toxoid and KLH. Laboratory studies will include measurement of IgM and IgG antibodies, and T-cell proliferation reponses before vaccines, and after (2 weeks, 1 month, 3 months, 6 months, 1 year).

C. CONCLUSIONS

The role of adhesion molecules and antibodies that interact through Fc receptors on NK cells has been explored. NK cell lysis of breast cancer targets is variable and is partially dependent on recognition through ICAM-1 and CD18; however, upregulation of ICAM-1 expression by cytokines does not enhance NK killing. While blocking CD2 slightly decreased cytotoxicity, contrary to expectations, an antibody against CD58 (the ligand for CD2), failed to block killing and instead mediated an increased cytotoxicity that correlated with target density of CD58. The CD58 antibody-enhanced killing was dependent not only on FcRγIII but also on CD2 and ICAM-1/CD18. To further elucidate the mechanism of this CD58 ADCC, Herceptin was tested. It mediated potent ADCC against all the HER2/neu positive breast cancer targets. Unlike CD58 antibody-mediated ADCC, Herceptin ADCC was minimally affected by blocking antibodies to CD2 or ICAM-1/CD18, which suggests a different mechanism of action. This study shows that multiple mechanisms are involved in NK cell lysis of breast cancer targets, that none of the targets are inherently resistant to killing, and that two distinct mechanisms of ADCC can target immunotherapy to breast cancer cells.

We feel that we have made significant progress in identifying and elucidating the mechanisms involved in IL-2 activated NK lysis of breast cancer targets. The ultimate goal of our studies is to translate our laboratory findings into clinical trials with therapeutic potential. Our data suggest that monoclonal antibodies combined with subcutaneous IL-2 may be a feasible method to increase the efficacy of immunotherapy against breast cancer, and is a strategy along with others that target immunotherapy which will be the focus of our future investigations.

D. APPENDICES

KEY RESEARCH ACCOMPLISHMENTS:

We have defined in great detail the immunologic mechanisms involved in IL-2 activated NK cell killing of breast cancer targets:

- NK cells require IL-2 activation to kill breast cancer targets.
- Upregulation of ICAM-1 surface expression by cytokines does not play a significant role in activated NK cell killing of breast cancer targets.
- CD58 antibody (AICD58) enhanced killing is dependent not only on FcRγIII (CD16).but also on CD2 and ICAM-1/CD18.
- Herceptin mediated killing is FcRγIII (CD16) dependent, yet relatively independent of CD2 and ICAM-1/CD18 expression.
- Two distinct mechanisms of ADCC can target immunotherapy to breast cancer cells.

REPORTABLE OUTCOMES:

1. Manuscript (preprint) is attached which details the results of our reserach to date. This manuscript is in press in Experimental Hematology.

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Experimental Hematology ■■ (1999) 1–9

Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu

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Treatment of advanced breast cancer with autologous stem cell transplantation is limited by a high probability of disease relapse. In clinical trials, interleukin 2 (IL-2) alone can expand natural killer (NK) cells in vivo and increase their cytotoxic activity against breast cancer cell lines, but this increase is modest. Understanding the mechanisms that mediate NK cell lysis of breast cancer targets may lead to improvements of current immunotherapy strategies. NK cells from normal donors or patients receiving subcutaneous IL-2 were tested in cytotoxicity assays against five breast cancer cell lines. The role of adhesion molecules and antibodies that interact through Fc receptors on NK cells was explored. NK cell lysis of breast cancer targets is variable and is partially dependent on recognition through ICAM-1 and CD18. While blocking CD2 slightly decreased cytotoxicity, contrary to expectations, an antibody against CD58 (the ligand for CD2), failed to block killing and instead mediated an increased cytotoxicity that correlated with target density of CD58. The CD58 antibody-enhanced killing was dependent not only on FcRγIII but also on CD2 and ICAM-1/CD18. To further elucidate the mechanism of this CD58 antibody-dependent cellular cytotoxicity (ADCC), another antibody was tested. Trastuzumab (Herceptin), a humanized antibody against HER2/neu, mediated potent ADCC against all the HER2/neu positive breast cancer targets. Unlike CD58 antibody-mediated ADCC, Herceptin ADCC was minimally affected by blocking antibodies to CD2 or ICAM-1/CD18, which suggests a different mechanism of action. This study shows that multiple mechanisms are involved in NK cell lysis of breast cancer targets, that none of the targets are inherently resistant to killing, and that two distinct mechanisms of ADCC can target immunotherapy to breast cancer cells. © 1999 International Society for Experimental Hematology. Published by Elsevier Science Inc.

Keywords: Natural killer cell—Antibody-dependent cellular cytotoxicity—Breast cancer—Interleukin 2—Immunotherapy

Introduction

Breast cancer incidence continues to increase among western women, with a 12% cumulative lifetime risk of developing the disease [1]. Although great progress has been made in patients with low-stage disease and favorable tumor characteristics, surgery, radiation therapy, and chemotherapy are still inadequate for high-risk or recurrent breast cancer. Autologous stem cell transplantation has been used as a treatment for breast cancer, but success is limited by a high rate of disease recurrence. Less than 40% of patients with good risk features obtain long-term disease-free survival, which suggests that preparative regimens are unable to eradicate all clonogenic tumor [2]. Results in patients with poor prognosis disease (organ involvement or chemotherapy resistance) are even worse, and long-term disease-free survival is rarely seen [3,4]. Although donor lymphocyte infusions have shown promising graft-vs-tumor effects in patients who relapse after allogeneic bone marrow transplantation [5], the potential of immunotherapy in patients with breast cancer remains unknown.

Natural killer (NK) cells are a phenotypically distinct population of lymphocytes ($CD56^+/CD3^-$) that were first identified by their ability to lyse tumor cells without prior immunization. They mediate both major histocompatibility (MHC)-independent and antibody-dependent killing of tumors and virally infected cells. Additionally, they proliferate and secrete cytokines on activation. Interleukin 2 (IL-2) activation of NK cells induces proliferation and increases cytotoxicity against a wide range of targets not susceptible to lysis by resting NK cells [6]. Antibody-dependent cellular cytotoxicity (ADCC) by NK cells is mediated by binding of FcRγIII (CD16) to the Fc portion of antibodies, which initiates a sequence of cellular events culminating in the release of cytotoxic, granzyme-containing granules [7]. Different signaling pathways are engaged in the process of natural cytotoxicity by which NK cells lyse susceptible targets such as tumor or virally infected cells [8]. Although NK cell killing is non-MHC restricted in that it does not require class I MHC for target recognition, NK cells express reper-

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toires of immunoglobulin-like killer inhibitory receptors, which recognize class I and may influence the balance of whether target cell lysis occurs by engaging an overriding inhibitory signal [9]. Although NK cells do not have antigen-specific receptors, the receptor/ligand pairs CD2/LFA-3 and LFA-1/ICAM-1 are involved in NK cell/target interactions [10,11]. Whether or not a target is killed by NK cells is determined by a net balance of these positive and negative signals [12]. To improve current immunotherapy, we investigated the mechanisms of NK cell recognition and lysis of breast cancer targets.

Materials and methods

Study population

Peripheral blood or marrow was obtained from normal donors or from patients after informed consent using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Peripheral blood mononuclear cells (PBMNC) or bone marrow mononuclear cells (BMMNC) were obtained by Ficoll-Hypaque (specific gravity 1.077) (Sigma Diagnostics, St. Louis, MO) density gradient centrifugation.

Normal NK populations

In initial studies to determine the effector specificity of breast cancer targets, PBMNC were sorted from the same donor for $CD4^+$ cells, $CD8^+$ cells, or $CD4^-/CD8^-$ cells. The CD4 and CD8 populations were cultured with irradiated mononuclear cells, 10 ng/mL OKT3 (Ortho Biotech, Raritan, NJ), and 1,000 U/mL IL-2 (a gift from Amgen, Thousand Oaks, CA) to yield greater than 98% pure populations of IL-2–activated $CD4^+$ or $CD8^+$ T cells. The $CD4^-/CD8^-$ population was cultured with irradiated mononuclear cells and 1,000 U/mL IL-2 to obtain an activated NK population devoid of T cells (<1%). In all subsequent studies, NK cells were enriched using a MACS column as specified by the manufacturer (Miltenyi, Auburn, CA). $CD56^+/CD3^-$ or $CD56^+/CD2^-$ NK cells were isolated by flow cytometry as described previously [6]. IL-2–activated NK cells were generated using PBMNC depleted of CD5/CD8 cells to enrich for NK cells and autologous monocytes as previously described [6] or by coculture of sorted $CD56^+/CD3^-$ NK cells on the murine stromal cell line, M210-B4 [13]. For both methods, NK cells were activated and expanded using an NK media supplemented with 1,000 U/mL IL-2 for 14 to 21 days prior to use. NK media consisted of a 2:1 (v/v) mix of DMEM/ Ham's F12-based medium (Gibco Laboratories, Grand Island, NY) supplemented with 24 μM 2-mercaptoethanol, 50 μM ethanolamine, 20 mg/L L-ascorbic acid, 5 μg/L sodium selenite (Na_2SeO_3), 100 U/mL penicillin, 100 U/mL streptomycin (Gibco), and 10% heat-inactivated human AB serum (North American Biologicals, Miami, FL) [14]. Resultant populations were greater than 90% $CD56^+/CD3^-$ cells.

NK populations from patients/treated with subcutaneous IL-2

PBMNC were obtained from patients enrolled on a clinical trial of posttransplant immunotherapy with daily subcutaneous IL-2 (1.75 MU/m^2/day; Chiron Therapeutics, Emeryville, CA). The details of the clinical trial eligibility and safety of the phase I study have been described [15]. Briefly, patients were eligible for immunotherapy when they were beyond 30 days after transplant, engrafted, off growth factors, transfusion independent, outpatients, free of infections, and had good performance status. Patient samples, enriched in vivo for NK cells by IL-2 therapy, were used as fresh PBMNC without further purification.

Generation of $CD56^+/CD16^-$ NK cells

$CD34^+/Lin^-/CD38^-$ cells were isolated from normal BMMNC as described and plated in NK medium in direct contact with the murine fetal liver cell line, AFT024 [16]. Progenitors were plated (1,000 cells/well) in 96-well plates and supplemented with 1,000 U/mL IL-2, 10 ng/mL flt3 ligand (FL; a gift from Immunex, Seattle, WA), 20 ng/mL c-kit ligand (KL or stem cell factor, a gift from Amgen), 20 ng/mL interleukin-7 (IL-7; R&D Systems, Minneapolis, MN), and a one-time addition at culture initiation of 5 ng/mL IL-3 (R&D Systems). Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 and the medium was half changed weekly with the indicated cytokines (without IL-3). After 5 weeks, cultures were transferred to T-25 flasks and cultured for an additional 2 weeks with IL-2 alone.

Cell lines

The human breast cancer cell lines were obtained from Dr. David Kiang (University of Minnesota, Minneapolis, MN). MCF-7 was cultured in modified Eagle's medium (MEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 0.2 μg/mL insulin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 1% sodium pyruvate. T47-D cells were cultured in RPMI 1640 (Gibco), supplemented with 10% heat-inactivated FCS, 0.1 μg/mL insulin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. SKBR-3, BT-20, and MDA-MB-231 were cultured in MEM (Gibco), supplemented with 10% heat-inactivated FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. In some experiments, cells were incubated with 1,000 U/mL interferon γ (Genzyme, Cambridge, MA). Cells were grown in monolayers in T-150 flasks (Corning, Cambridge, MA) at 37°C in a humidified atmosphere with 5% CO_2 prior to use.

Cytotoxicity, immunophenotyping, and antibodies

Cytotoxicity assays were performed at the indicated effector to target ratios (E:T) using resting or IL-2–activated NK cells against cell lines in a 4-hour ^{51}Cr release assay [17]. Monoclonal antibodies against CD58 (IgG2a, AICD58; Immunotech), CD58 (IgG2a, BRIC-5, Biosource), ICAM-1 (IgG1, 84H10, Immunotech), HER2/neu (IgG1, 2G11, Biosource), Trastuzumab (Herceptin, a humanized IgG1 antibody, Genentech, Inc., San Francisco, CA), and control IgG2a (X39, Becton Dickinson, San Jose , CA) were added to targets. CD18 (IgG3, P4H9, Gibco), CD2 (IgG2a, S5.2; Becton Dickinson), and CD16 (IgG1, 3G8, Immunotech) were added to the NK cells. All antibodies were added at a concentration of 10 μg/mL unless otherwise indicated 30 minutes prior to each assay and remained for the duration of the 4-hour incubation. Phenotype analyses were performed with a FACSCalibur (Becton Dickinson) and CELLQuest software (Becton Dickinson) using antibodies ICAM-1-PE (Becton Dickinson), CD58-PE (Immunotech), HER2/neu-FITC (Biosource), CD56-PE (Becton Dickinson), and CD16-FITC (Becton Dickinson).

Statistics

Results of experimental points obtained from multiple experiments were reported as mean ± 1 SEM. Significance levels were determined by two-sided Student's *t*-test.

Results

NK killing of breast cancer targets

The ability of NK cells to lyse breast cancer targets was assessed in vitro. Because IL-2 alone cannot efficiently expand human NK cells in vitro, coculture with autologous monocytes or stromal feeders was used. The NK expansion after 14 to 21 days using either of these two methods (NK cells cocultured with monocytes [6] or NK cells cocultured on the M210-B4 cell line [13]) is between 50- and 200-fold. These expanded populations have greater NK cell purity (>90% $CD56^+/CD3^-$) than traditional lymphokine-activated killer cells, which contain a heterogeneous mixture of NK cells and T cells. Resting purified $CD56^+/CD3^-$ NK cells from normal donors (E:T 6.6:1) exhibited low lytic activity against all of the breast cancer targets (less than 10% specific lysis, n = 4, data not shown). In contrast, activation and expansion of NK cells with 1,000 U/mL IL-2 and accessory cells resulted in an increase in cytotoxicity against all breast cancer targets. The cytotoxicity was mediated solely by the $CD56^+/CD3^-$-activated NK cells, and bulk IL-2–activated CD^{4+} or CD^{8+} T cells did not contribute to target lysis [data shown for MCF-7 (Fig. 1A)]. These activated NK populations exhibited significant but variable cytotoxicity against five breast cancer cell lines (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3). All of these cell lines, with the exception of BT-20, were originally derived from pleural effusions of patients with metastatic breast cancer [18]. The MCF-7, T47D and MDA-MB-231 targets were consistently more sensitive to activated NK cell lysis compared to the BT-20 or SKBR-3 targets, which were killed less efficiently (Fig. 1B).

Role of β2 integrin/ICAM interactions in NK cell killing of breast cancer targets

β2 Integrin (CD18) recognition of ICAM-1 is a described mechanism of recognition for NK-mediated killing of various fresh and cultured tumor targets [18,19]. To test the role of this recognition mechanism against breast cancer targets, several experiments were performed. Breast cancer targets were evaluated for ICAM-1 expression by flow cytometry after culture in their respective media with or without the addition of interferon γ, a known inducer of ICAM-1 expression [20]. SKBR-3, a cell line killed less efficiently by activated NK cells and with low basal expression of surface ICAM-1, significantly increased ICAM-1 expression after a 24-hour preincubation of targets with interferon γ (from a mean channel fluorescence [MCF] of 440 to 818). SKBR-3 targets then were tested in cytotoxicity assays to determine whether the increased ICAM-1 expression increased their susceptibility to activated NK cell lysis. In contrast to our hypothesis, interferon γ treatment of targets made them more resistant to lysis despite the increase in ICAM-1 surface expression (data not shown), which suggests that other factors play a role in determining target sensitivity.

The contribution of β2 integrin/ICAM-1 interactions toward the lysis of breast cancer targets was assessed directly in experiments with blocking antibodies against ICAM-1, CD18, or the combination. Consistent with data shown in Figure 1B, the baseline lysis of MCF-7 targets was highest and lysis was not significantly inhibited with any of the single antibodies or combinations tested (data not shown). In contrast, blocking antibodies alone or in combination variably inhibited lysis of the remaining breast cancer targets (Fig. 2). The combination of antibodies resulted in greater inhibition than single antibodies, except for SKBR-3 where CD18 blocking and the combination of CD18 and ICAM-1 resulted in similar inhibition. There was no significant difference in target lysis inhibition with ICAM-1 blocking for the breast cancer targets with the highest surface ICAM-1 expression (MDA-MB-231 [MCF = 891], BT-20 [MCF = 799]) and the targets with the lowest expression (T47D [MCF = 264], SKBR-3 [MCF = 440]). Furthermore, sur-

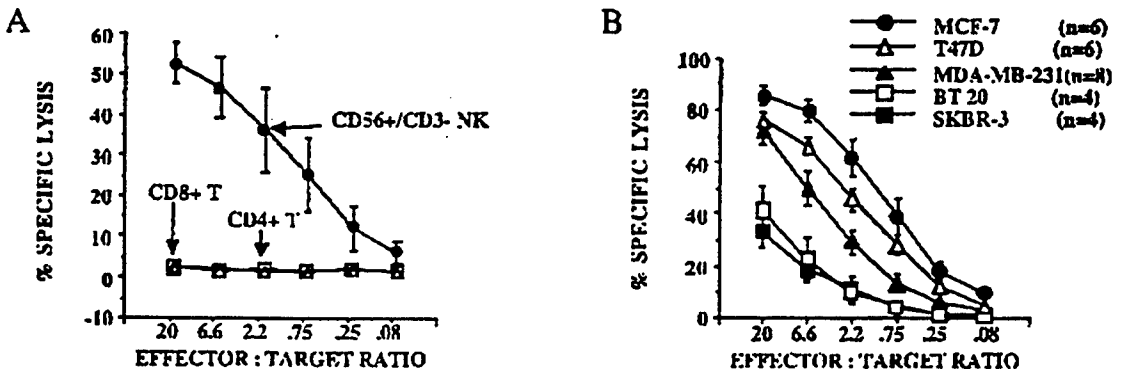

Figure 1. IL-2–activated NK cells mediate significant cytotoxicity against breast cancer targets in vitro. (A) IL-2–activated populations of $CD4^+$, CD^{8+}, and NK cells were all generated from blood of normal donors and tested in cytotoxicity assays against the MCF-7 target. Activated NK cells (92 ± 2% $CD56^+/CD3^-$), but not $CD4^+$ or $CD8^+$ T cells, exhibited lysis of MCF-7 targets (n = 3). (B) NK cells and autologous monocytes were obtained from normal donors and activated for 14 to 18 days with 1,000 U/mL IL-2. The resultant populations (90 ± 3% $CD56^+/CD3^-$) were tested in cytotoxicity assays against five breast cancer cell lines (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3).

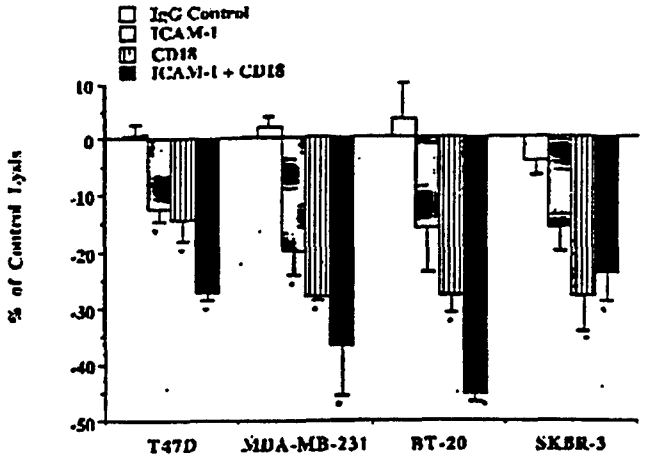

Figure 2. ICAM-1/CD18 interactions are involved in IL-2–activated NK lysis of breast cancer targets. IL-2–activated NK cells (96 ± 2% $CD56^+/CD3^-$) from normal donors were tested in cytotoxicity assays against breast cancer targets at an effector to target ratio of 4:1. Specific lysis was calculated for each target in the absence of antibody for T47D (79 ± 3.4%), MDA-MB-231 (70 ± 2.7%), BT-20 (45 ± 9.3%), and SKBR-3 (53 ± 9%). Data are presented as the percent of control with each antibody or combination as follows: [(% lysis with antibody − % lysis without antibody)/(% lysis without antibody)]. Each bar represents mean ± SEM of 4 to 6 individual experiments analyzed in duplicate. There was significant inhibition of specific lysis for each breast cancer target involving recognition through β2 integrins on NK cells. $^*p < 0.05$.

face expression of ICAM-1 did not correlate with sensitivity to killing.

Role of CD2/LFA-3 interactions in NK cell killing of breast cancer targets

In addition to β2 integrin recognition of targets, the interaction of CD2 on NK cells with CD58 on some targets has been described [11,21]. To test the role of this ligand pair, experiments were performed using antibodies in cytotoxicity assays to determine their effect on breast cancer target cell lysis. Addition of anti-CD2 antibodies to NK cells resulted in less than 10% change in specific lysis, which was not significantly different from controls without antibody or with mouse IgG. In contrast, addition of the CD58 antibody (AICD58) to targets consistently increased killing of MDA-MB-231, BT-20 and SKBR-3 (data not shown). Addition of the CD58 antibody (AICD58) alone to targets without effectors did not result in lysis, which suggests that CD58 antibody may be functioning through ADCC.

Table 1. Mean channel fluorescence of CD58 and HER2/neu on breast cancer cell lines

Cell line	CD58(LFA-3)	HER2/neu
MCF-7	153	62
T47D	57	83
MDA-MB-231	147	54
BT-20	286	105
SKBR-3	90	1,676

Mean channel fluorescence (MCF) of the isotype control was between 6 and 22 for all samples.

Antibodies against CD58 mediate ADCC

Breast cancer cell lines were phenotyped for surface expression of CD58 and HER2/neu, a known antigen overexpressed on some breast cancers. All targets were positive for CD58 and HER2/neu with variable expression (Table 1). BT-20 expressed the highest surface density of CD58, whereas T47D was the least positive. This correlated well with the increased killing of BT-20 by IL-2–activated NK cells. HER2/neu expression was highest on SKBR-3 and lowest on MDA-MD-231. There was no apparent correlation between the relative expression of CD58 and HER2/neu expression between the breast cancer targets tested.

Resting $CD56^+/CD3^-$ NK cells from normal donors were purified using flow cytometry and tested without further activation in cytotoxicity assays against all five breast cancer targets. In addition to the CD58 antibody that increased lysis with IL-2–activated NK cells, another CD58 clone (BRIC-5, IgG2a) was tested. As expected, the baseline killing of breast cancer targets by NK cells without IL-2 activation was low at E:T 10:1. There was no difference in lysis between assays performed without antibody as compared to those with IgG control or with the CD58 (BRIC-5)

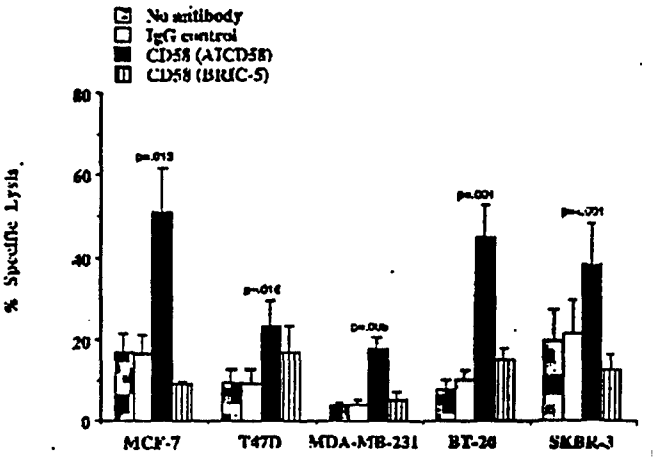

Figure 3. Incubation of breast cancer targets with CD58 (AICD58) antibody enhances antibody-dependent cellular cytotoxicity. Resting NK cells were purified from normal donors by flow cytometry (>97% $CD56^+/CD3^-$) and incubated with breast cancer targets at an effector to target ratio of 10:1 without activation with IL-2. Cytotoxicity was performed without (n = 6) or with the addition of mouse IgG (n = 6), anti-CD58 (clone AICD58, IgG2a, n = 6), or anti-CD58 (clone BRIC-5, IgG2a, n = 2). Each bar represents the percent specific lysis (mean ± SEM) of experiments analyzed in duplicate. The CD58 (AICD58) antibody significantly enhanced lysis of all breast cancer targets as indicated compared to the mouse IgG control. No other differences were found.

antibody. In contrast, the CD58 (AICD58) antibody significantly increased killing against all breast cancer targets tested (Fig. 3). The fold increase in the mean specific lysis for the cell lines with the highest CD58 expression (MCF-7, MDA-MB-231, BT-20) was greater than the fold increase in mean specific lysis for the breast cancer cell lines with the lowest CD58 expression (T47D, SKBR-3). Titration experiments with the CD58 (AICD58) antibody and the BT-20 target showed enhanced lysis down to an antibody concentration of 0.1 μg/mL (n = 2).

In vivo activated mononuclear cells collected from patients treated with subcutaneous IL-2 exhibited relatively low cytotoxicity against all the breast cancer cell lines. MCF-7, which is consistently the most susceptible to lysis by activated NK cells, is not lysed by patient mononuclear cells collected prior to starting IL-2 therapy (2.4 ± 1.1% lysis at E:T 60:1). Twenty-eight days of subcutaneous daily IL-2 treatment induced a modest increase in cytotoxicity by patient mononuclear cells against MCF-7 (19 ± 3.1% lysis at E:T 60:1) and less of an increase against other cell lines (data not shown). To measure the effect of the CD58 (AICD58) antibody on NK cells expanded in vivo, we tested mononuclear cells from patients receiving subcutaneous IL-2 against BT-20, the breast cancer target with the highest expression of CD58. Specific lysis of BT-20 targets was approximately 10% when tested without antibody or with an isotype-matched IgG2a control antibody. Similar to the results obtained with normal donor cells, addition of the CD58 (AICD58) antibody increased target lysis to 60% (Fig. 4, left), six times greater than control. This demonstrates that NK cells expanded in vivo with IL-2 express functional Fc receptors.

To assess whether the CD58 (AICD58) antibody-enhanced killing was dependent on Fc receptors, populations of CD16 (FcRγIII) negative NK cells were generated from IL-2–dependent, stromal-dependent long-term culture using marrow-derived $CD3^{4+}$ progenitors cells. $CD56^+/CD3^-$ NK cells were generated from $CD34^+/Lin^-/CD38^-$ cells that coexpressed 1.1 ± 0.3% CD16 (n = 4). In contrast to the normal donor NK cells and the in vivo activated patient mononuclear cells used in the previous assays, the IL-2–activated NK progeny ($CD56^+/CD16^-$) derived from marrow progenitors did not mediate ADCC (Fig. 4, right).

Blocking antibodies were used to determine which accessory molecules were necessary for the enhanced killing by the CD58 (AICD58) antibody. Both mononuclear cells from patients receiving IL-2 (data not shown) and normal donor purified NK cells (n = 4) were tested against the breast cancer target BT-20. Cytotoxicity assays were performed with CD58 antibody alone and in combination with ICAM-1, CD18, CD2, and CD16. Blocking ICAM-1/CD18 interactions, CD2 alone or CD16 alone significantly inhibited the enhanced killing by the CD58 (AICD58) antibody, and the combinations completely abrogated the enhanced effect.

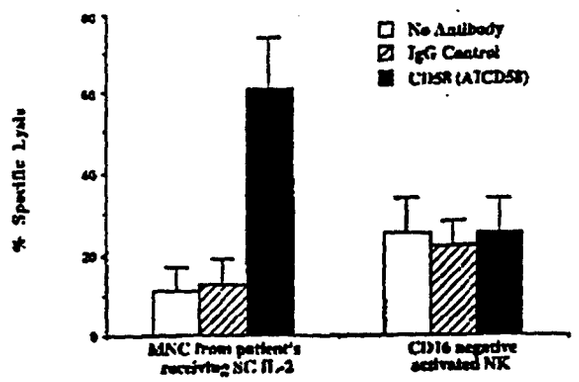

Figure 4. CD58-mediated antibody-dependent cellular cytotoxicity (ADCC) increases lytic activity in mononuclear cells (MNC) from patients receiving subcutaneous (SC) IL-2 but not in NK cells, which are CD16 negative. Peripheral blood mononuclear cells were obtained from patients treated for 14 to 28 days with SC IL-2 (1.75×10^6 U/m²/day) on a protocol to prevent relapse after autologous stem cell transplantation. Patient mononuclear cells (63 ± 8% $CD56^+/CD3^-$, of which 87 ± 2% expressed FcRγIII [CD16]), without further IL-2 activation, were tested against the BT-20 breast cancer target at an effector to target ratio of 20:1. Cytotoxicity was significantly enhanced ($p < 0.05$, n = 4 patient samples performed in duplicate) when targets were pretreated with CD58 (AICD58) antibody compared to no antibody or IgG control. The requirement for FcRγIII (CD16) on NK cells was tested further by generating NK cells from marrow $CD34^+$ progenitors in a long-term NK cell differentiation culture. IL-2 cultured NK cell progeny (97 ± 1% $CD56^+/CD3^-$, of which 1.1 ± 0.2% expressed FcRγIII [CD16]) were tested against BT-20 at an effector to target ratio of 10:1. Unlike normal donor or patient-derived NK cells, the $CD16^-$ NK cells derived from marrow progenitors were unable to mediate ADCC when targets were pretreated with the CD58 (AICD58) antibody (n = 4).

Trastuzumab (Herceptin) mediates ADCC through a different mechanism

If the CD58 antibody was mediating classic ADCC by signaling through FcRγIII, the significant blocking effect of CD2 would remain unexplained. To further explore this finding, we tested another antibody that mediates ADCC. Herceptin is a humanized antibody against HER2/neu engineered by inserting the complementary determining regions of a murine antibody (clone 4D5) into the framework of a consensus human IgG1 [22]. In contrast to the HER2/neu murine antibody (clone 2G11, IgG1), which did not mediate ADCC (n = 2, data not shown), Herceptin added to normal $CD56^+/CD3^-$ NK cells significantly enhanced killing of all breast cancer targets except for MDA-MB-231, the target with the lowest HER2/neu expression (Fig. 5). Titration experiments with the Herceptin antibody and the SKBR-3 target, the target with the highest expression of HER2/neu, showed enhanced lysis down to an antibody concentration of 0.01 μg/mL (n = 2), which was the concentration used in subsequent ADCC blocking experiments. Marrow-derived $CD16^-$ NK cells did not augment killing of SKBR-3 targets in the presence of Herceptin (n = 6, data not shown). Similar to CD58 (AICD58) ADCC, Herceptin augmented killing by resting blood NK cells was also FcRγIII (CD16) dependent as

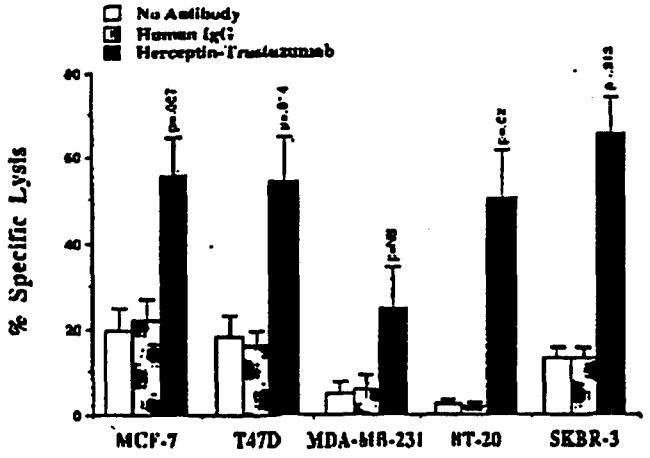

Figure 5. Incubation of breast cancer targets with Herceptin (humanized anti-HER2/neu) mediates antibody-dependent cellular cytotoxicity. Resting NK cells were purified from normal donors and incubated with breast cancer targets at an effector to target ratio of 10:1 without activation with IL-2. Cytotoxicity was performed without antibody or with the addition of human IgG or Herceptin at 10 μg/mL (n = 4 in triplicate). The Herceptin antibody significantly enhanced lysis of all breast cancer targets except MDA-MB-231, as indicated. Cytotoxicity with Herceptin is compared to the human IgG control.

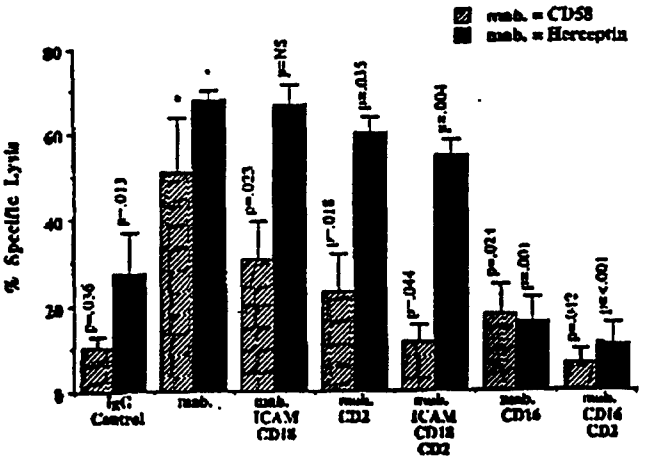

Figure 6. CD58 antibody-mediated antibody-dependent cellular cytotoxicity (ADCC) but not Herceptin-mediated ADCC is decreased by blocking ICAM-1/CD18 and CD2. Sorted (>97% $CD56^+/CD3^-$) normal donor NK cells (effector to target ratio 10:1), without further IL-2 activation, were tested against the BT-20 (hatched bars) or SKBR-3 (black bars) breast cancer targets along with antibodies that mediate ADCC, CD58 (hatched bars) and Herceptin (0.01 μg/mL, black bars), respectively. Cytotoxicity was performed in the presence or absence of blocking antibodies as indicated (n = 4 donors in triplicate). Both the CD58 (AICD58) antibody and Herceptin significantly increased lytic activity compared to IgG control. In the presence of CD58 antibody, addition of antibodies to block ICAM-1, CD18, and CD2 resulted in suppression of CD58 antibody-mediated ADCC, whereas Herceptin ADCC decreased only slightly. All *p* values listed are compared to the addition of monoclonal antibody (mab) alone (*).

shown using blocking antibodies (Fig. 6). In contrast to CD58 (AICD58) ADCC, which was decreased by nearly 50% by CD2 or ICAM-1/CD18, these same blocking antibodies had less of an effect on Herceptin ADCC (Fig. 6). Whereas blocking both CD2 and ICAM-1/CD18 completely abrogated CD58 (AICD58) ADCC, ADCC with Herceptin was only slightly blocked with the same combination of antibodies.

Although both antibodies [CD58 (AICD58) and Herceptin] result in CD16-dependent killing, blocking experiments suggest different interactions with accessory receptor/ligand pairs. CD58 (AICD58)-mediated ADCC appears to be CD2 dependent, whereas Herceptin ADCC is minimally affected by blocking CD2. To further test this, we used a subset of NK cells that is CD56 and CD16 positive but CD2 negative. This subset, which generally comprises 10 to 40% of normal blood NK cells [23], was purified by flow cytometry (Fig. 7A). Secondary staining of $CD56^+/CD2^-$ sorted NK cells showed that greater than 80% expressed CD16. $CD56^+/CD16^+/CD2^-$ NK cells were still able to augment target lysis of Herceptin-treated SKBR-3 targets, which suggests a CD2-independent mechanism of ADCC signaling through CD16. In contrast, $CD56^+/CD16^+/CD2^-$ NK cells did not lyse CD58 (AICD58) antibody-treated BT-20 targets, which confirms the CD2 dependence of this ADCC and the lack of triggering through CD16 alone (Fig. 7B).

Discussion

Breast cancer relapse remains a major clinical problem even after dose-intensive therapy such as autologous transplantation. We hypothesize that immunotherapy used in a minimal residual disease setting after transplantation may serve as a noncross-resistant therapy to prevent relapse. Although NK cells are among the first immune effectors to reconstitute after stem cell transplantation, resting NK cells do not exhibit activity against breast cancer targets until they are activated with exogenous IL-2. We are concerned that well-tolerated doses of IL-2 alone may not be efficacious.

Normal NK cells were found to exhibit variable killing of five breast cancer cell lines, whereas bulk CD^{4+} or CD^{8+} T cells exhibited no activity. SKBR-3, the target with the highest HER2/neu expression, was the most resistant to IL-2–activated NK lysis, as has been observed by others [24]. The role of ICAM-1 in effector recognition of targets has been studied extensively, and it seemed reasonable to hypothesize that sensitivity to lysis may correlate with the relative expression of ICAM-1 on targets. This notion was supported by data from Budinsky et al. [19], who found that primary breast cancer cells expressed lower ICAM-1 than benign breast tissue, suggesting that tumors may escape immune recognition by decreasing their ICAM-1 expression. However, our results and those of others do not support this premise. There was no correlation between surface expression of ICAM-1 and target sensitivity to NK cell lysis, and induction of ICAM-1 on targets failed to make them more

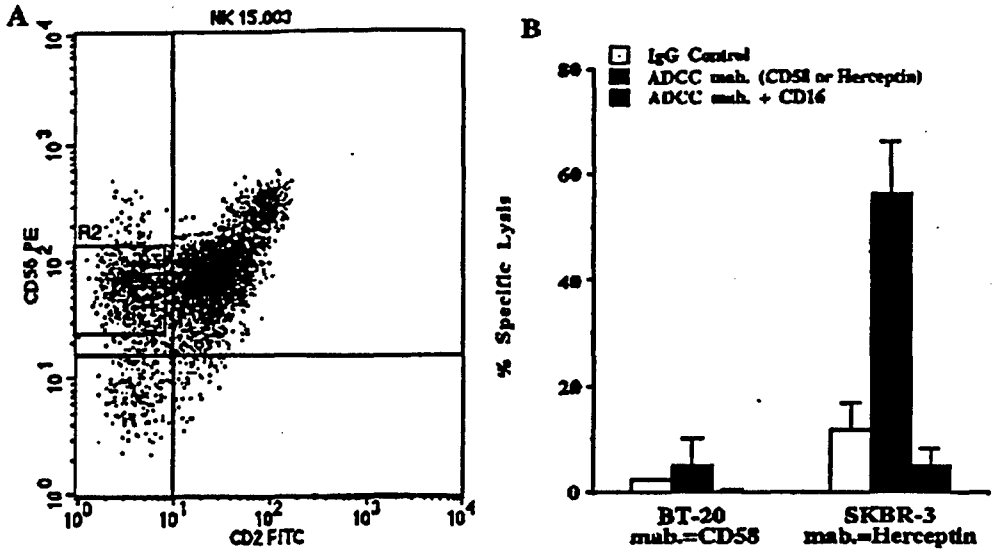

Figure 7. CD58-mediated antibody-dependent cellular cytotoxicity (ADCC) but not Herceptin-mediated ADCC is dependent on CD2. (A) $CD56^+/CD2^-$ NK cells were sorted by flow cytometry. An example of a representative sort with the collection window designated R2 is shown. (B) $CD56^+/CD2^-$ NK cells, which are predominantly CD16 positive, were tested in cytotoxicity assays using the breast cancer target and antibodies as indicated. The CD2- NK cells exhibited CD16-dependent killing of Herceptin-treated SKBR-3 targets but had no effect on CD58 (AICD58) antibody-treated BT-20 targets.

susceptible. These results do not exclude ICAM-1 as playing an important role in NK killing. They merely suggest that ICAM-1 interactions are among the many factors determining target cell lysis. In agreement with our data, Gwin et al. [21] showed that increased ICAM-1 expression by interferon on A-375 (melanoma) and Daudi (lymphoma) tumor cells increased effector/target conjugation but decreased killing. ICAM-1 may be more important for initial recognition, whereas other interactions and postbinding events further modulate the fate of whether a target is killed [20]. The concurrent upregulation of class I MHC, also induced by interferon γ, may explain this observation. This is of particular interest in light of the multiple structures and variants of class I recognizing receptors found on all NK cells, although their physiologic relevance in cancer is still uncertain.

The combination of ICAM-1 and CD18-blocking antibodies did not result in greater than 50% inhibition for any of the targets, which suggests that other mechanisms were operant. This led to experiments exploring the role of CD2/CD58 in the lytic mechanism. Although interrupting the cell/target interaction with antibody to either CD2 [25] or CD58 [11,21] has been shown to inhibit target cell lysis, the CD58 (AICD58) antibody used here mediated the opposite effect and enhanced target killing. Consistent with ADCC, the CD58 (AICD58) antibody effects were independent of IL-2 activation and NK cell CD16 (FcRγIII) was required in the process. We used unique differences between mature NK cells and those derived from long-term cultures of marrow progenitors to generate NK cells that were CD16 negative. We have shown that these cells exhibit characteristic lysis of K562 targets demonstrating that their lytic machinery is intact [26,27]. The failure of the CD58 (AICD58) antibody to enhance killing by the marrow progenitor-derived NK cells demonstrates a requirement for CD16, which is consistent with Fc-mediated ADCC. The ability to generate functional NK cells lacking specific receptors can be a useful tool to dissect the complex interactions involved in NK cell killing.

ADCC by NK cells is mediated through binding of IgG immune complexes or antibody-coated targets to the low-affinity Fc receptor for IgG, FcRγIII. The α subunit of CD16, which binds the Fc portion of IgG molecules, associates noncovalently with the signal-transducing molecules CD3ζ and FcεRI-γ [7]. It is thought that antigen density and structure, as well as the isotype specificity of Fc binding, all contribute to the induction of ADCC [28]. Human NK cells have been shown to exhibit ADCC using murine antibodies of several isotypes (IgG1, IgG2a, IgG2b, IgG3) [29, 30]. Others report a variation among individual donors in the NK response to IgG of different isotypes [31]. We found clone-specific anti-CD58–mediated ADCC. As both CD58 antibodies were isotype IgG2a, the inability of clone BRIC-5 to mediate ADCC may be due to epitope specificity or to some characteristic of tertiary structure. Similarly, we were not able to induce ADCC with anti-HER2/neu antibody clone 2G11 (IgG1), whereas several others have described ADCC using different clones of the same isotype [22,32].

Accessory cell molecules may play an important role in CD58 (AICD58) antibody-mediated ADCC. In addition to the primary role for CD16, our data also show a role for

ICAM-1/LFA-1 interaction in ADCC similar to that described by Lanier et al. [9]. We could not find any reports of ADCC mediated through CD58. However, antibodies against its ligand, CD2, have been shown to activate NK lysis when cross-linked to Fc receptor positive targets, a process called reverse ADCC or antibody-redirected lysis [9]. The finding that antibody against CD2 blocks CD58 (AICD58) antibody-mediated ADCC suggests that CD16 and CD2 may be colocalized on the NK cell surface. The CD58 antibody, linked to the NK cell Fc receptor (CD16), may serve as an anchor to increase the affinity of CD2 to its natural ligand. Blocking CD2 may sterically hinder this association, which suggests that the signal for target lysis may be through CD2 rather than CD16, as described for classic ADCC. Direct evidence to support this notion was obtained from experiments using a normal NK cell subset that is $CD56^+/CD16^+/CD2^-$. These $CD2^-$ NK cells were unable to enhance target lysis with the CD58 (AICD58) antibody, proving that CD16 alone is insufficient to mediate this mechanism of ADCC. This is contrasted to ADCC mediated by Herceptin, which exhibits classic ADCC through CD16, which is CD2 independent. Both antibodies still mediate ADCC when titrated down to low concentrations and recognize targets with a broad range of either LFA-3 or HER2/neu surface densities.

We have been studying whether IL-2–based immunotherapy has an anti-tumor effect that can be used as additional adjuvant therapy after stem cell transplantation to increase survival in patients with breast cancer. We have previously shown that subcutaneous IL-2 can be given safely to autologous transplant patients and that daily IL-2 in vivo expands NK cells that exhibit increased cytotoxicity against breast cancer targets [15]. Despite these promising results, the in vivo activity induced by subcutaneous IL-2 therapy after transplantation is submaximal when compared to NK cells activated ex vivo with a higher concentration of IL-2, which raises the possibility that current therapy may be insufficient to mediate a therapeutic response. We show that ADCC with the CD58 (AICD58) or Herceptin antibodies markedly enhances killing of breast cancer targets by two different mechanisms: one through CD16 signaling and one by increasing the affinity of a receptor (CD2) to its natural ligand (CD58). Our data suggest that monoclonal antibodies combined with subcutaneous IL-2, which expands NK cells 10-fold in vivo without loss of Fc function as shown here, may be a feasible method to increase the efficacy of immunotherapy against breast cancer. This strategy and others that target immunotherapy will be the focus of future investigations.

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REPLY TO
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MCMR-RMI-S (70-1y) 26 Aug 02

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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